J. Anat. (1992) 180, pp. 41–56, with 4 figures Printed in Great Britain

Cytochemical localisation and characterisation of proteoglycans (glycosaminoglycans) in the epithelial–stromal interface of the seminal vesicle of the guinea pig

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(*Accepted 12 August 1991*)

ABSTRACT

The proteoglycans (PGs) in the guinea pig seminal vesicle were demonstrated ultrastructurally by both cuprolinic blue (CB) and ruthenium red (RR) staining. The PGs appeared as electron-dense granules with RR, but were filamentous following CB staining using the critical electrolyte concentration method. Three major types of PGs (T1, T2, T3) have been described according to their different locations and sizes. T1 filaments were short and were found mostly on both sides of the lamina densa of the basal lamina of the glandular epithelium (40–60 nm long) and also on the basal laminae of smooth muscle cells and capillary endothelial cells (20–30 nm long). In the epithelial basal lamina they were regularly spaced at an interval of 40–60 nm. T1 filaments in the lamina densa were smaller and more randomly distributed. Cytochemical characterisation of these PGs by various GAG degrading enzymes showed that T1 PGs are rich in heparan sulphate. T2 filaments were 30–40 nm long and closely associated with the collagen fibrils. They were arranged perpendicular to the long axis of collagen fibrils, also at intervals of about 60 nm. T2 filaments were removed by chondroitinase (Ch)-ABC, Ch-ABC plus *Streptomyces* (S)-hyaluronidase and pronase, but resistant to nitrous acid, heparitinase, heparinase, neuraminidase and S-hyaluronidase. These show that T2 filaments are rich in dermatan sulphate. T3 filaments (60–100 nm) were widely distributed in the stroma at sites such as the interstitial spaces of the lamina propria, the reticular layer below the basal lamina, around individual collagen fibrils or bundles of such fibres, and on the cell surfaces of fibroblasts. The T3 filaments were removed by Ch-ABC, Ch−AC and pronase but were resistant to heparitinase, heparinase, S-hyaluronidase, neuraminidase and nitrous acid. They are therefore rich in chondroitin sulphate.

INTRODUCTION

Proteoglycans (PGs) are large extracellular molecules each consisting of a core protein to which the glycosaminoglycans (GAGs) chains and various O-linked and N-linked oligosaccharide chains may be covalently attached (Hascall and Hascall, 1981).

PGs are important components of the extracellular matrix and basement membrane but they can also be associated with the cell membrane either as integral or pericellular components (Kjellen et al. 1980, 1981; Nordling et al. 1981; Rapraeger and Bernfield, 1983, 1985; Bernfield et al. 1984; Rapraeger et al. 1985, 1986; Hayashi et al. 1987). PGs are not now considered to be passive, inert molecules and they are known to play an important role in maintaining the structural, physical and biological properties of extracellular matrices. They also play a role in regulating the interactions between cells and their environment (Hook et al. 1984), which are important for normal cell growth and development, and for the maintenance of cellular function (Bissell et al. 1982; Hay, 1984). Because of their polyanionic nature, they can inhibit or regulate the diffusion of other macromolecules across basement membranes, control the access of important regulatory molecules to the cell surface such as growth factors and hormones, and influence local cation balance, especially that of Ca^{2+} (Obrink et al. 1975; Kanwar, 1984; Lerner and Torchia, 1986; Thomas and Gimenez-Gallego, 1986;

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Ingber et al. 1987; Roberts et al. 1988). Those PGs located at the epithelial–stromal interface, including the basal lamina, have been shown to be involved in a number of cellular phenomena such as cell–matrix interactions, cell migration, cell morphology, and growth and branching morphogenesis of exocrine glands (Tool, 1981; Bernfield et al. 1984; Hay, 1984; Reddi, 1984). These effects are primarily due to the polyanionic nature of PGs, their expanded configuration in tissues and their ability to interact with a variety of extracellular matrix components. Alterations in PGs have been detected in a number of pathological processes such as malignancy, atherosclerosis and inflammation. These qualitative and quantitative changes in PG composition may significantly influence the behaviour of proliferating cells by producing a hydrated environment which favours cell migration and growth (Iozzo, 1985).

Surprisingly little is known of the role of PGs and their metabolism in the normal and abnormal growth of male accessory sex glands. Sato & Gyorkey (1972) have studied prostatic GAGs in relation to the zinc content of the normal human prostate. They demonstrated biochemically that CS and DS are the predominant GAGs. DeKlerk (1983), DeKlerk et al. (1984) and DeKlerk & Human (1985) have also conducted a number of biochemical studies on GAGs in fetal and pubertal human prostate, and benign hyperplastic and cancerous prostates. Four types of GAGs: chondroitin sulphate (CS), dermatan sulphate (DS), heparan sulphate (HS) and hyaluronic acid (HA) have been identified biochemically in the human prostate, although no HS was detected in fetal prostate (DeKlerk, 1983). The stromal GAGs have been shown to change during prostate development (DeKlerk & Human, 1985). The levels of DS, HS and HA sulphation appeared to correlate directly with known periods of increased serum levels of testosterone and epithelial growth. Increased amounts of DS and HS were shown to occur during adulthood, suggesting that prostatic epithelial proliferation at puberty may be associated with changing GAG levels. The level of CS increases both in benign prostatic hypertrophy and prostatic carcinoma, while HS and its sulphation are decreased in prostatic carcinoma (DeKlerk et al. 1984). The GAG content appears to fluctuate with changes in hormonal milieu. The concentration of GAGs in the seminal vesicle and prostate of the rat has been shown to change (the level of uronic acid increases) on castration and testosterone administration after orchidectomy (Kofoed et al. 1971). Rzeszowska (1966) has shown histochemically that the acid mucopolysaccharides (or GAGs) in the seminal vesicle are reduced after castration or oestradiol treatment, but increased by exogenous testosterone administration.

Chan and Wong (1989*a*, *b*) have recently shown that in the guinea pig lateral prostate 3 different classes of PGs can be identified. Those associated with the basal lamina are mainly HS, while those attached to stromal collagen fibrils are DS. CS PGs are largely found in the interstitial space. The present report described the localisation and characterisation of PGs in the seminal vesicle of the guinea pig.

MATERIALS AND METHODS

Cuprolinic blue and ruthenium red staining

Fifteen adult male guinea pigs weighing 700–800 g were used in this study. The animals were perfused with either (1) 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4 (before RR staining), or (2) 2.5% glutaraldehyde in 0.025 M sodium acetate buffer at pH 5.6 (before CB staining). The mildly prefixed seminal vesicles were excised and trimmed into small blocks. The tissue blocks were processed further either by the cuprolinic blue (CB; BDH Ltd, UK) or the ruthenium red (RR; Taab, UK) staining procedures as outlined previously (Chan & Wong, 1986*b*). After staining, the tissues were dehydrated with ethanol and embedded in Epon. Thin sections were cut, stained with uranyl acetate and lead citrate and examined either with a Philips EM 300 at 60 kV or a JEOL EM 2000 FX electron microscope at 80 kV.

Enzymes, nitrous acid and $MgCl_2$ treatments

The procedures for treatment with different glycosides, nitrous acid and $MgCl_2$ were the same as described previously (Chan & Wong, 1989*a*). The detailed conditions of different treatments are listed in Table 1. For enzyme and $MgCl_2$ treatments, the fresh unfixed tissue blocks were incubated in different enzyme solutions or 2.0 M $MgCl_2$ in 0.025 M sodium acetate buffer for various times. For nitrous acid digestion, the tissues were prefixed in 2.5% glutaraldehyde in 0.025 M sodium acetate buffer at pH 5.6 for 2 h before incubating in nitrous oxide. Protease inhibitors, 5 mM benzamidine-HCl and 0.1 M, 6-amino-*n*-caproic acid (Sigma Chemical Co., USA), were added to all incubation solutions in order to prevent any nonspecific protease digestion (Hedman et al. 1983; Van Kuppevelt et al. 1984*b*). After the enzyme, $MgCl_2$ or nitrous acid treatments, the tissues were reacted by the CB staining procedure at the

Table 1. *Enzymatic and chemical treatments used for characterisation of cuprolinic blue-positive filaments*

Treatment*	Concentration	Buffer – pH	Duration/ temperature	Reference
Chondroitinase ABC (*Proteus vulgaris*)	U/ml	0.25 M Tris, 0.18 M NaCl, 0.05% BSA, pH 8.0	1–3 h at 37 °C	Saito et al. (1968)
Chondroitinase AC (*Arthrobacter aurescens*)	1 U/ml	0.25 M Tris, 0.18 M NaCl, 0.05% BSA, pH 8.0	1–3 h at 37 °C	Saito et al. (1968)
Heparinase I (*Flavobacterium heparinum*)	5 U/ml	0.1 M sodium acetate, 0.15 M NaCl, pH 7.0	1–3 h at 37 °C	Linker & Hovingh (1972)
Heparitinase + heparinase II (*Flavobacterium heparinum*)	5 U/ml	0.1 M sodium acetate, 0.15 M NaCl, pH 7.0	1–3 h at 43 °C	Linker & Hovingh (1972)
Hyaluronidase (*Streptomyces hyaluroly-ticus*)	50 U/ml	0.1 M sodium acetate, 0.15 M NaCl, pH 5.6	90 min, 2, 3 h at 37 °C	Saito et al. (1968)
Chondroitinase ABC + *Streptomyces* hyaluronidase	1 U/ml + 50 U/ml	—	2, 3, 24 h at 4 °C	—
Neuraminidase (*Clostridium perfringes*)	1 U/ml	0.1 M sodium acetate, 0.15 M NaCl, pH 5.4	1–2 h at 37 °C	Kanwar & Farquhar (1979*c*)
Pronase + protease type XIV (*Streptomyces griseus*)	15 U/ml	0.25 M Tris, 0.18 M NaCl, 0.05% BSA, pH 7.4	45 min, 2 h at 37 °C	—
Nitrous acid	33% acetic acid + 5% $NaNO_2$ mixed 1:1	—	2, 3, 24 h at 4 °C	Cifonelli (1968)
$MgCl_2$	2.0 M	0.025 M sodium acetate, pH 5.6	2 h at 4 °C	Van Kuppevelt et al. (1984*b*)

* All enzymes were purchased from Sigma Chemical Co., St Louis, MO.

critical electrolyte concentration (CEC) (Scott, 1980) for the PG demonstration.

RESULTS

Ultrastructural localisation of proteoglycans

Two staining procedures were applied to demonstrate PGs (or GAGs) at the ultrastructural level: (1) staining with CB using the CEC method, followed by in block staining with sodium tungstate; (2) staining with RR.

Cuprolinic blue staining

PGs (or GAGs) were visualised as various electron-dense filaments after staining with CB according to Scott's (1980) CEC method. They appeared as dense filaments at high concentrations of $MgCl_2$ (0.3, 0.4 M), while at low concentrations of $MgCl_2$ (0.025 M), in addition to the filamentous forms, granular electron-dense particles were sometimes seen. The results presented here were based on observations at high $MgCl_2$ concentration (0.3 M). According to their sizes and locations, 3 different types of CB-stained filaments could be distinguished. They were designated as type 1 (T1), type 2 (T2) and type 3 (T3) filaments.

Like those reported in the guinea pig lateral prostate (Chan & Wong, 1989*a*), T1 filaments were observed in the basal laminae of the glandular epithelium, smooth muscles and capillary endothelium of the seminal vesicle (Figs 1–3). In the epithelial basement membrane, they were mostly located on both sides of the lamina densa, and some could also be seen within the lamina densa. Those filaments located in the lamina lucida and on the stromal side of the lamina densa appeared to be more electron-dense and larger in size and number than those in the lamina densa. The former had a length of 40–60 nm while those in the lamina densa, which usually appeared granular, measured 10–20 nm in diameter. These filaments appeared to be in a plane parallel to the lamina densa and equally separated from each other. Those T1 filaments in the lamina lucida were separated from each other by an interval of 40–60 nm, while those on the stromal side of the lamina densa were separated by 80–100 nm in CB-stained specimens.

T1 filaments were also demonstrated in the basal lamina of vascular endothelium. Those located in the lamina lucida and on the stromal side of the lamina densa appeared to be larger (~ 30 nm by CB), while those in the lamina densa were granular and smaller (10 nm by CB and RR), and less electron-dense. The latter were randomly distributed in the lamina densa. T1 filaments seen in the basal lamina of the smooth muscle cells were located mainly in the lamina densa (Fig. 3). They appeared to be smaller (~ 20 nm by CB) than those located in the epithelial basal lamina. The lamina lucida of the smooth muscle cell basal lamina was usually not well defined.

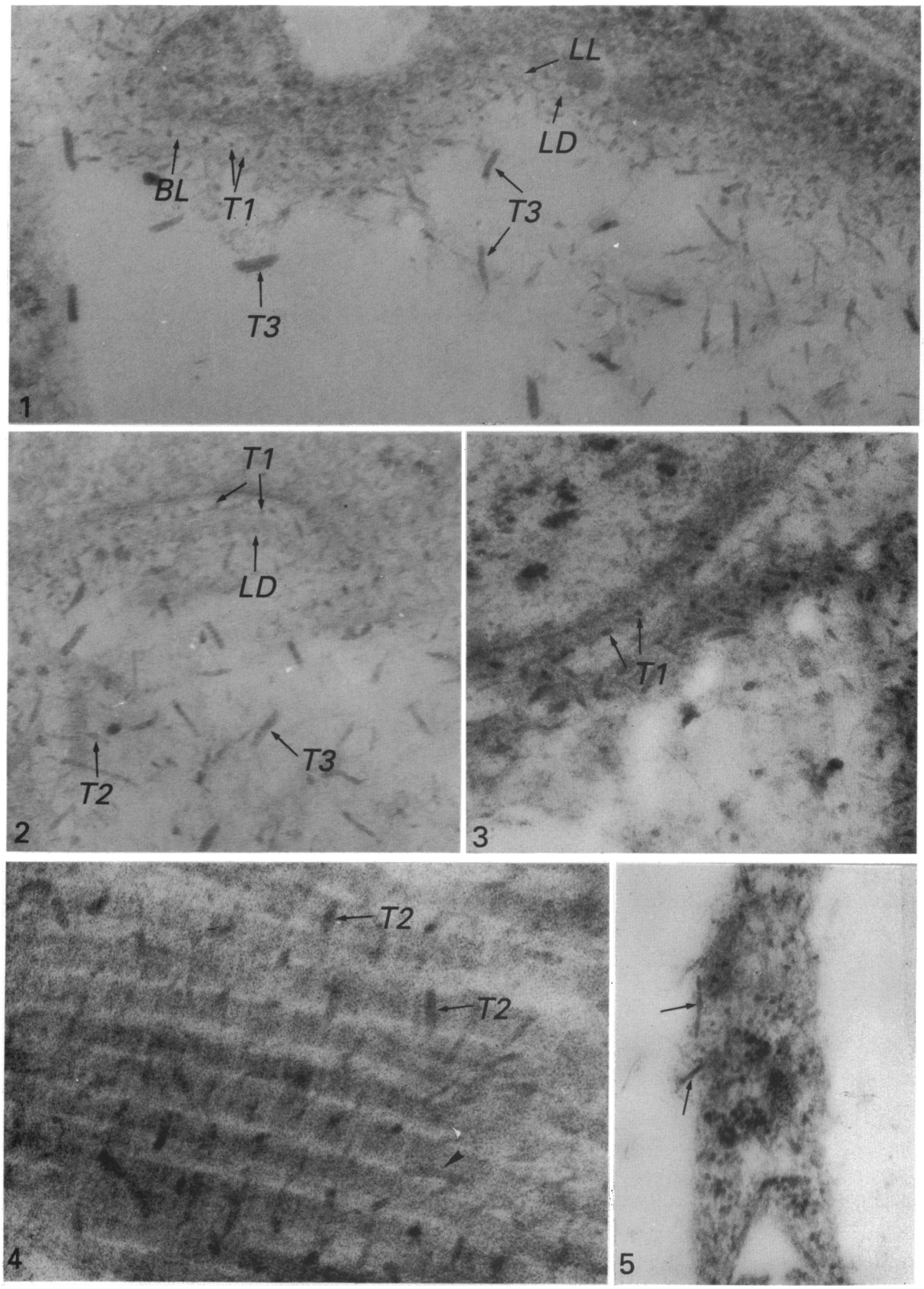

Fig. 1. Transmission electron micrograph of seminal vesicle stained with cuprolinic blue (CB) in 0.3 M $MgCl_2$, showing CB-T1 (*T1*) filaments in the basal lamina (*BL*) of the epithelial cell). The T1 filaments are seen arranged regularly in the lamina lucida (LL) and the stromal side of the lamina densa (*LD*). Note the T3 (*T3*) filaments in the reticular layer. × 66 500.

Fig. 2. Another region in the epithelial basal lamina showing again T1 filaments in the basal lamina, T3 filaments in the interstitial space and T2 filaments closely associated with collagen fibrils. × 66 500.

Fig. 3. The basal lamina of smooth muscle cell appears to have only a single layer of T1 filaments on the outer surface of the lamina densa. × 69 000.

Fig. 4. T2 CB-filaments are seen closely associated with collagen fibrils (*T2*). Note that some CB filaments are lying parallel to the long axis of collagen fibrils (arrowhead). × 148 000.

Fig. 5. Micrograph showing the CB-T3 filaments closely associated with the surface of a fibroblast (arrows). Fine filaments are also seen projecting from the CB filaments. × 50 000.

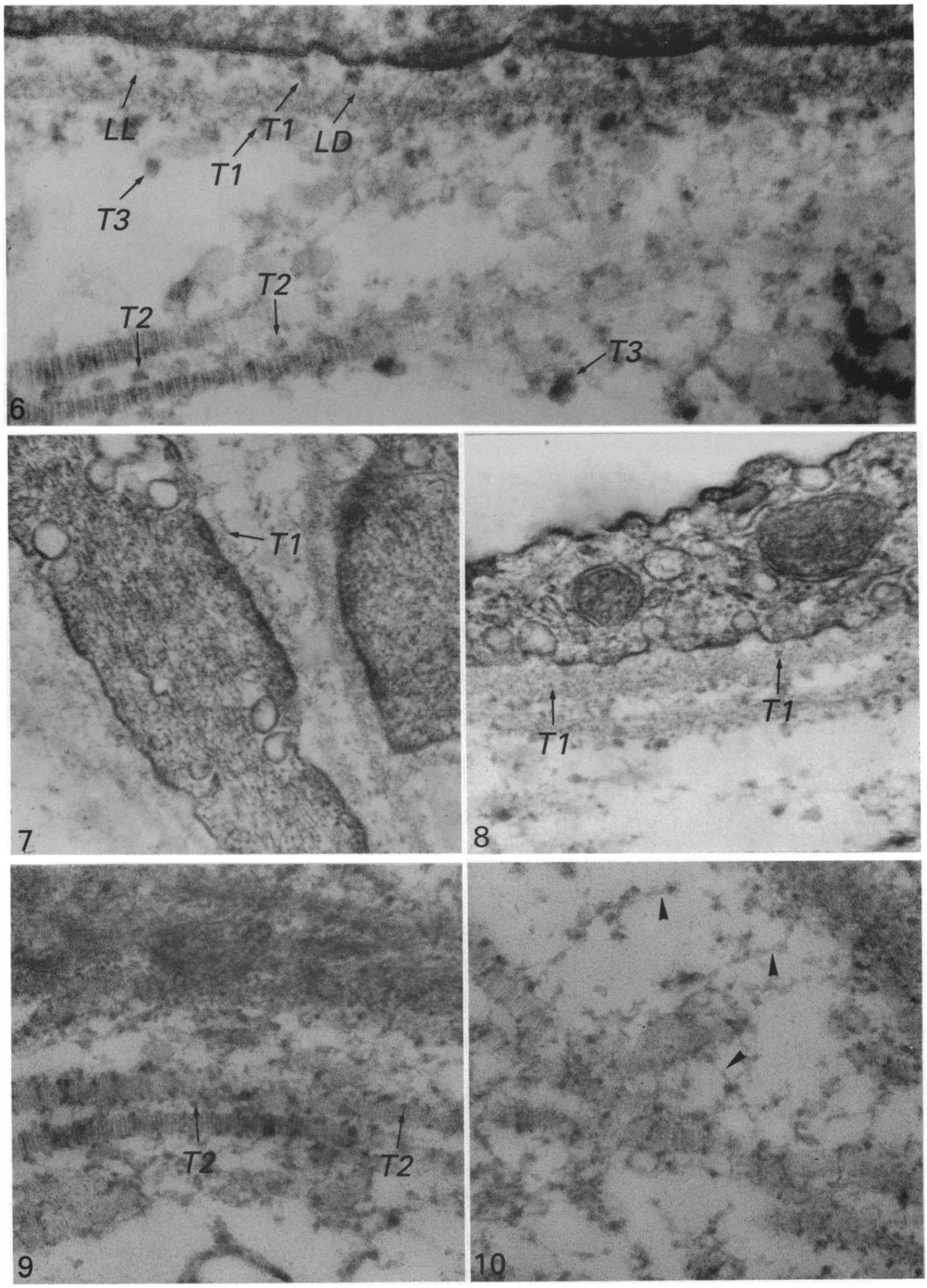

Fig. 6. Micrograph of seminal vesicle tissue stained with ruthenium red (RR). RR type 1 granules (*T1*) are located mainly on the lamina lucida (*LL*) and the stromal side of lamina densa (*LD*) of the epithelial basal lamina. They are separated from each other by an interval of 60–70 nm. The T2 and T3 granules (*T2*, *T3*) are also found in the reticular layer. × 130000.

Fig. 7. The T1 granules (*T1*) are demonstrated in the lamina densa of the smooth muscle. They appear to be smaller (~ 10 nm) and less electron-dense than those located in the epithelial basal lamina. × 72000.

Fig. 8. The T1 RR-granules (*T1*) are located on both sides of the lamina densa and the lamina densa of the capillary endothelium. × 70000.

Fig. 9. The T2 PGs associated with the collagen fibrils ar stained as granules (*T2*) by RR. These granules are seen lying regularly on the surfaces of collagen fibrils. × 74000.

Fig. 10. Micrograph showing the PGs after RR staining. Fine networks of filaments (arrowheads) connecting the PG granules to one another, to collagen fibrils or to the basal lamina are clearly shown. × 73000.

T2 filaments measuring 40 nm in length were seen to be closely associated with the collagen fibrils (Fig. 4). The patterns of PG–collagen fibril interaction were better shown with the use of the CB/CEC method. The CB-positive filaments lay perpendicularly to the long axis of fibrils and were separated from each other by an interval of 60 nm. At higher magnification, the filaments were found to be linked to the D band of the collagen fibril. Some CB-stained filaments (40–45 nm in length) were seen lying parallel to the fibrils (Fig. 4).

Finally, the third type (T3) of CB-stained filaments was heavily stained and of variable size. These filaments measured 60–80 nm in length. They were located in several places in the lamina propria: (1) in the reticular layer (pars fibroreticularis) below the basal lamina (Fig. 1); (2) at the surface of collagen fibrils (Fig. 2); (3) in the interstitial spaces of the lamina propria; and (4) at the cell surface of fibroblasts (Fig. 5). Fine filaments were sometimes seen projecting from the CB-stained filaments.

Ruthenium red staining

By RR fixation and staining, the PGs (or GAGs) were revealed as electron-dense polygonal granules. According to their sizes and locations, 3 main types of RR-stained granules were again observed. They were accordingly designated as T1, T2 and T3 granules (Fig. 6). Their distribution and locations resembled the filaments (T1, T2 and T3) stained by CB.

The RR-stained T1 granules, measuring about 10–20 nm in diameter, appeared on both sides of the lamina densa of the basal lamina. They were regularly spaced with an interval of about 40–60 nm between them in both the lamina lucida and the stromal surface of the lamina densa. The RR-stained granules located in the lamina lucida were sometimes seen connected by very fine filaments ($\sim$ 3 nm) to the epithelial plasmalemma on one side and the lamina densa on the other. Such a structural relationship was also demonstrated between the RR-stained granules on the stromal side of the basal lamina and the lamina densa. The RR-stained granules were also found in the basal laminae of the smooth muscle (Fig. 7) and endothelial cells (Fig. 8).

T2 granules, measuring 10–20 nm in diameter, were found closely associated with the collagen fibrils (Figs 6, 9). They were seen regularly arranged and separated from each other, with an interval of about 60 nm corresponding to the D band period of the collagen fibrils.

T3 granules were present in different sites in the lamina propria, and their sizes ranged from 30 to 60 nm. At times several large electron-dense RR-stained granules formed chains linked together by a fine filament ($\sim$ 5 nm) (Fig. 10). In addition, T3 granules were also observed on the surface of fibroblasts, around bundles of collagen, particularly the loosely packed collagen fibrils, and in interstitial spaces adjacent to the epithelial basal lamina.

These RR-stained granules could still be seen when the tissues were fixed in the same concentration of RR in 0.1 M veronal–acetate–HCl buffer at pH 2.5, thus indicting the presence of sulphate groups in the RR-positive binding sites, because carboxyl groups are hardly ionised at such a low pH.

Cytochemical characterisation of cuprolinic blue-stained proteoglycans in the seminal vesicle

CB-positive filaments were characterised by treating the tissues with a number of GAG-degrading enzymes and nitrous acid. A summary of the effects of the various treatments on the CB-positive filaments is given in Table 2.

Basement membrane CB-positive filaments. The CB-positive T1 filaments localised in the basal laminae of secretory epithelium, vascular endothelium and smooth muscle cells were no longer visible after treatment with heparitinase (Fig. 11) and nitrous acid (Figs 12, 13). Neither heparitinase nor nitrous acid could remove completely the CB-stained filaments on the stromal side of the lamina densa of the capillary basal lamina (Figs 12, 14). Moreover, digestion with chondroitinase (Ch)-ABC (Fig. 15), Ch-AC (Fig. 16), heparinase (Fig. 20), neuraminidase (Figs 17, 18) and *Streptomyces* hyaluronidase (Fig. 19) had no effect on these T1 filaments, but they were partly removed after treatment with 2.0 M $MgCl_2$ without prefixation (Fig. 21). After digestion of tissue with pronase, without prefixation, no filaments could be detected. These results suggest that the CB-positive filaments in the basal laminae of the seminal vesicle are HS-containing PGs.

Collagen fibril-associated CB filaments. The collagen-associated T2 filaments disappeared after digestions with Ch-ABC (Fig. 15) and pronase. Treatment with nitrous acid, heparinase, heparitinase, neuraminidase and *Streptomyces* hyaluronidase had no effect on the CB–T2 filaments (Figs 1, 12, 17, 19, 20), nor could Ch-AC completely remove them (Fig. 16). Furthermore, treatment of the tissue with 2.0 M $MgCl_2$ before fixation partly removed T2 filaments (Fig. 21). These results indicate that the CB–T2 filaments are PGs rich in iduronic acid containing dermatan sulphate.

Table 2. *Effects of various enzymatic treatments on CB-stained proteoglycans in seminal vesicles*

Treatment	Specificity	Basement membrane filaments (T1)	Collagen-associated filaments (T2)	Interstitial filaments (T3)
Chondroitinase ABC	Chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate	+	−	−
Chondroitinase AC	Chondroitin sulphates, hyaluronic acid and dermatan sulphate which contains a glucuronic acid	+	±	−
Nitrous acid	Heparan sulphate and heparin	−	+	+
Heparinase	Heparin	+	+	+
Heparitinase	Heparan sulphate	−	+	+
Neuraminidase	Sialic acid	+		
Streptomyces hyaluronidase	Hyaluronic acid	+	+	+
Chondroitinase ABC + *Streptomyces* hyaluronidase	Hyaluronic acid chondroitin sulphate and dermatan sulphate	+	−	−
Pronase	Proteins	−	−	−
2.0 M $MgCl_2$	Breaks ionic bonds	±	±	−

−, CB filaments unaffected
±, CB filaments partially affected
+, CB filaments absent

Interstitial CB filaments. The CB-positive T3 filaments were no longer detectable after digestion with Ch-ABC, Ch-AC and pronase (Figs 15, 16). The filaments were resistant to treatment with nitrous acid, heparitinase, heparinase, S-hyaluronidase and neuraminidase (Figs 13, 17, 19). Extraction with 2.0 M $MgCl_2$ removed most of the T3 filaments (Fig. 21). This indicates that the T3–CB filaments represent PGs containing CS.

DISCUSSION

There have been no systematic studies on the ultrastructural localisation and characterisation of PGs in the male accessory sex glands of any species of mammal including man. Chan & Wong (1989*a*, *b*) have recently demonstrated the localisation of PGs of the lateral prostate of the guinea pig. The present study has demonstrated at the electron microscopic level the distribution of the GAGs/PGs at the epithelial–stromal interface of the seminal vesicle of the guinea pig. They appear to have different shapes depending on the kinds of dyes and methods employed. They appeared as filamentous forms by CB using the CEC method, but as granules when RR was used. The precise mechanisms of reactions between CB or RR with PGs are not known. It is believed that with the CEC method the CB dyes are held together by Van der Waals forces to form a complex which acts as a scaffold binding stoichiometrically to the polyanionic GAG chains, so forming an extended complex which collapses on to the core proteins of the PGs. This renders the PGs visible as filaments (Scott, 1985).

As for RR staining, the visualisation of the PGs is believed to result from the condensation of the GAG chains on to the coiled core protein to form dense granules of different sizes depending on the length of the GAG chains (Hascall, 1980). In contrast to CB, RR molecules are not held together to form a complex.

As has been shown by Chan & Wong (1989*b*) in the prostate gland, the 3 types of filaments (T1–T3) resulting from CB staining are analogous with the 3 sizes of granules (also designated as T1–T3) in RR-stained specimens. The differences in sizes and location of these PGs suggest that they may differ in chemical composition and their role in the tissues.

Basal lamina PGs

The results showed that PGs are present in the basal laminae of the glandular epithelia, smooth muscle and endothelia of the seminal vesicle. They were present mostly on both sides of the lamina densa of the epithelial and endothelial basal laminae. However, in the basal lamina of the smooth muscle cells the T1 PGs lay mainly in the lamina densa. The basal lamina PGs visualised by CB and RR appeared more or less similar in size in the prostate gland and seminal vesicle. However, differences in size and electron density were observed between those in the lamina lucida (40–60 nm by CB; larger and denser) and lamina densa (10–20 nm by CB) of the epithelial basal

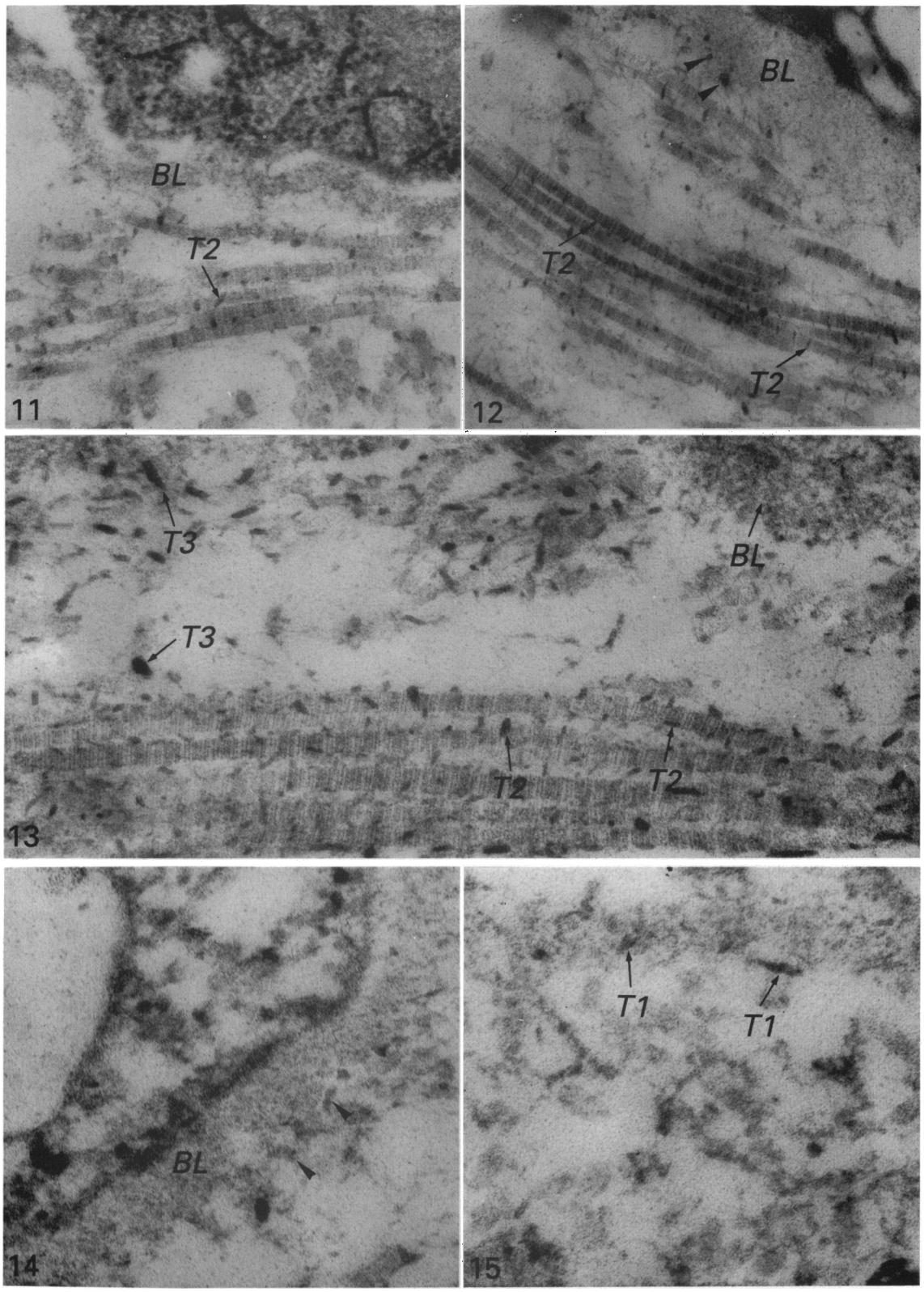

Fig. 11. Heparitinase digestion. Heparitinase digestion completely removes the CB-T1 filaments in the basal lamina (*BL*) of epithelial cells. However, heparitinase has no effect on the CB-T2 filaments (arrows). × 59000.

Fig. 12. Same treatment as in Figure 11, but showing the basal lamina of capillary endothelium. Note that a few CB filaments (arrowheads) can still be seen on the stromal side of the lamina densa. The T2 filaments are not affected by heparitinase digestion. × 50000.

Fig. 13. HNO_2 treatment. The results are identical to heparitinase digestion. HNO_2 eliminates all CB-T1 filaments in the basal lamina (*BL*) of glandular epithelium. CB-T2 and T3 filaments are unaffected. × 84800.

Fig. 14. HNO_2 treatment. Note a few CB-T1 filaments (arrowheads) on the stromal side of the lamina densa of the capillary basal lamina (*BL*). × 100000.

Fig. 15. Chondroitinase-ABC digestion. Ch-ABC has no effect on the CB-T1 filaments (*T1*), while CB-T3 filaments in the interstitial spaces and around collagen fibrils as well as the T2 filaments are removed. × 125000.

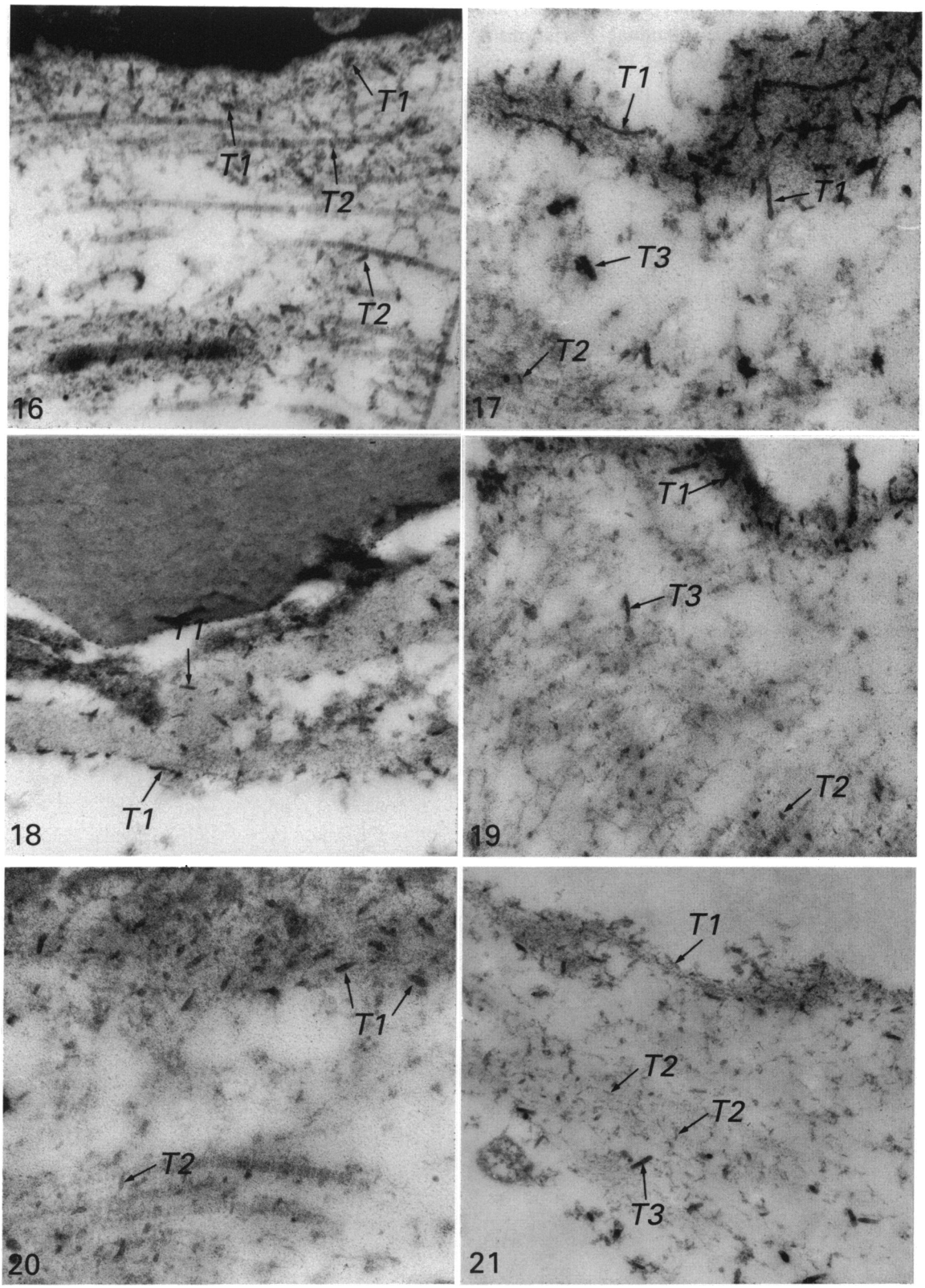

Fig. 16. Chondroitinase-AC treatment. The T2 filaments (*T2*) remain after chondroitinase AC digestion. The T3 filaments are not seen in the reticular layer. Note also the T1 filaments (*T1*), which still remain in the basal lamina. × 54000.

Fig. 17. Neuraminidase digestion. Neuraminidase has no effect on any of the types of epithelial CB-filaments (*T1*, *T2*, *T3*). × 57000.

Fig. 18. The T1 filaments of capillary endothelium persist after neuraminidase digestion. × 42500.

Fig. 19. *Streptomyces* hyaluronidase digestion has no effect on any of the 3 types of CB filaments. × 42500.

Fig. 20. Heparinase I digestion. The T1 and T2 filaments can still be seen after heparinase digestion (T1 and T2). × 60000.

Fig. 21. $MgCl_2$ extraction. High electrolyte concentration (20 M $MgCl_2$) does not completely remove all types of CB filaments (*T1*, *T2*, *T3*). × 34000.

lamina and between those in the epithelial (larger) and smooth muscle (smaller) basal laminae. Variations in the size and shape of CB-stained PGs in the same basal lamina have been described in the basal lamina of human endothelial cells and rat parietal yolk sac (Iozzo, 1985; Iozzo & Clark, 1987). Biochemical studies have shown heterogeneity of molecular size within the same basal lamina. Two different forms of PGs (low-density and high-density forms) have been isolated from the basement membrane of the EHS (Engelbreth–Holm–Swarm) tumour (Fujiwara et al. 1984; Dziadek et al. 1985; Hassell et al. 1985). They are shown to differ in their core molecular species and GAG chain structures (Kato et al. 1987).

It is of interest to note that the T1 filaments (and also T1 granules) in the lamina lucida, as well as on the stromal side of the lamina densa, are regularly separated from each other by an interval of about 40–60 nm in the former and 80–100 nm in the latter. The regular repeating arrangement of stained PGs is not unique to the seminal vesicle. It has been described in the lamina lucida of a number of basement membranes of tissues such as cornea (Trelstad et al. 1974), embryonic salivary gland (Cohn et al. 1977; Spooner & Paulsen, 1986), glomerulus (Kanwar & Farquhar, 1979*a*), pulmonary alveolus (Vaccaro & Brody, 1979; Van Kuppevelt et al. 1984*a*, 1985), mammary gland (Gordon & Bernfield, 1980), and prostate gland (Chan & Wong, 1989*a*). These specific regular arrays of PGs in the lamina lucida indicate the highly ordered organisation of the basement membrane components, which consist of type IV collagen, laminin, HSPGs and entactin/nidogen (Martinez-Hernandez & Amenta, 1983; Martin & Timpl, 1987). Type IV collagen, located mainly in the lamina densa, forms a continuous network to which other components are attached. HSPGs have been shown to bind specifically to both laminin and type IV collagen (reviewed by Yurchenco et al. 1987; Martin & Timpl, 1987). At high magnification, very fine filaments were seen to project from the RR-positive T1 granules. The nature of these very fine linking filaments is not known. Similar fine strands have been described crossing the lamina lucida and reaching the cell membrane (Inoue & Leblond, 1988). As PGs can interact with other BM components, such as type IV collagen and laminin, it is possible that these fine filaments may represent these components. These filaments join the RR-stained granules to the epithelial cell plasma membrane and the lamina densa.

Cytochemical characterisation revealed that the CB-stained T1 filaments of the basal laminae of the glandular epithelia, capillary endothelium and smooth muscle cells of the seminal vesicle represent PGs rich in HS. The present results are consistent with those found in the lateral prostate of the guinea pig (Chan & Wong, 1989*b*) and most of the previous reports.

PGs are now recognised as important components of the basement membrane. HSPGs have been shown to be the major type of PGs found in most basement membranes of normal tissues. They have been demonstrated cytochemically in the basal laminae of various tissues such as the renal glomerulus (Kanwar & Farquhar, 1979*a*, *c*; Laurie et al. 1984), mammary epithelium (Gordon & Bernfield, 1980), lung alveolus (Vaccaro & Brody, 1979; Van Kuppevelt et al. 1984*b*, 1985), endothelium (Clowes et al. 1984; Ausprunk, 1986) and prostatic epithelium (Chan & Wong, 1989*b*). HSPGs have been isolated and characterised biochemically from a variety of basement membranes such as kidney glomerular basement membrane (Kanwar & Farquhar, 1979*b*; Kanwar et al. 1981; Parthasarathy & Spiro, 1981) and basement membrane-producing tumours (EHS tumour) and tumour cell lines (Oldberg et al. 1979; Hassell et al. 1980; Oohira et al. 1982; Fenger et al. 1984; Fujiwara et al. 1984). However, CSPGs have also been demonstrated and isolated from some basement membranes such as mouse embryonic salivary gland (Cohn et al. 1977), human term placenta (Parmley et al. 1984), fenestrated capillaries (Simionescu et al. 1984), murine Reichert's membrane (Paulsson et al. 1985; McCarthy et al. 1989), rat embryonic parietal yolk sac (Iozzo & Clark, 1986, 1987), and rat Bowman's capsule, epidermis and skeletal muscle (McCarthy et al. 1989). Hybrid PGs with both HS and CS side chains are found on mouse mammary epithelial cells (Rapraeger & Bernfield, 1985) and EHS tumour (Kato et al. 1987).

HSPGs of basement membranes of the capillary and smooth muscle appeared to be smaller in size and less electron-dense than those of the epithelium. This may indicate a difference in the type of HSPGs present in different basement membranes. The difference in their electron density may be a reflection of differences in the sulphate content of the GAG chains, while the difference in length of the CB-stained filaments may be due to the difference in molecular weight of the core protein. The fact that they still persisted after extraction by 2.0 M $MgCl_2$ indicated that they were firmly attached to the basement membrane.

The functions of basement membrane PGs are still not well understood, and some functions have been postulated including selective filtration in renal glomeruli and capillaries (Kanwar et al. 1980; Kanwar, 1984), maintenance of normal structure and function of tissue (Martin et al. 1984; Paulsson et al. 1986;

Martin & Timpl, 1987), interactions with growth factors (Thomas & Gimenez-Gallego, 1986; Ingber et al. 1987; Roberts et al. 1988) and extracellular ions (Obrink et al. 1975; Lerner & Torchia, 1986), formation of basement membrane (Laurie, 1985) and regulation of cellular differentiation and development of glandular morphogenesis (Cohn et al. 1977; Bernfield et al. 1984; Bernfield, 1984; Hay, 1984; Li et al. 1987).

Collagen-associated PGs

T2 PGs, 20–40 nm in diameter on CB staining, have been shown to be associated with collagen fibrils in the stroma. Two types of associations between the CB-stained PGs and collagen fibrils were observed: orthogonal (perpendicular) and axially parallel. Most of the stained PGs were seen lying perpendicular to the long axis of collagen fibrils. In CB–CEC-treated specimens, some T2 filaments were seen running parallel to the collagen fibrils. Like the T1 filaments in the basal lamina, perpendicularly arranged T2 filaments were also evenly spaced at intervals of 60 nm, very close to the D-band period (64 nm) of the collagen fibrils. This regular spacing of the filaments along the length of collagen fibrils is probably due to their binding to a specific band of the collagen. Such an orthogonal arrangement of PGs on collagen fibrils has been described following the AB–CEC staining method (Ruggeri et al. 1975; Schofield et al. 1975). Scott and Orford (1981) and Scott et al. (1981), using the CB–CEC method, have demonstrated the close association of PGs with the a–e bands of collagen fibrils in developing rat tendon. Thus the PGs are located at the gap zones of the fibrils. Our present study confirms such a relationship of the T2 filaments with the D (a–e) band of the collagen fibrils. However, the nature of the interaction of the parallel arranged T2 filaments to the collagen fibrils is not certain. It is known that PGs play a role in maintaining the interconnection between collagen fibrils, hence the mechanical properties of the collagen fibrils (Danielson, 1982). They are also known to regulate collagen fibril formation and their diameters by regulating the extracellular condensation of tropocollagen molecules (Obrink, 1973; Scott & Orford, 1981; Flint et al. 1984).

The CB-positive T2 filaments were removed by digestion with Ch-ABC, Ch-ABC plus S-hyaluronidase, and pronase. They were partly affected by Ch-AC. However, they were insensitive to nitrous acid, heparitinase, heparinase, neuraminidase and S-hyaluronidase. The results indicate that T2 filaments are PGs containing iduronic acid-rich DS. The partial removal by Ch-AC suggests that they may also contain CS or DS of glucuronic acid. DS can be considered as an isomer of CS in which a variable proportion of D-glucuronic acid is converted to L-iduronic acid by an epimerase. However, it is shown that the formation of L-iduronic acid is generally incomplete, and thus DS chains are hybrid molecules with variable disaccharide units of either type (Fransson & Havsmark, 1970; Malmstrom et al. 1975). The proportion of disaccharide units containing L-iduronic acid varies widely. This explains the variable effect of Ch-AC on T2 filaments.

DSPGs are widely distributed in tissue and occur in relatively high concentration in fibrous connective tissues such as skin, tendon, aorta, sclera, joint capsule and cornea (Scott et al. 1981; Heinegard & Paulsson, 1984; Iozzo & Clark, 1986), which contain predominantly type I collagen. It has been shown that GAGs interact electrostatically with type I collagen under physiological conditions (Scott, 1988). DS, with a higher charge density than CS, has been shown to bind more strongly to collagen fibrils than CS and can thus increase the structural and mechanical strength of the collagen fibrils (Danielson, 1982). PGs are important in the process of fibrillogenesis. DSPGs and CSPGs have been shown to inhibit fibril growth in vitro when incorporated into type I collagen (Vogel et al. 1984) and DSPGs have been shown to be able to precipitate collagen monomers from solution (Obrink, 1981). They may therefore play a key role in the regulation of collagen fibril assembly and the mechanical strength of collagen fibrils. During tissue maturation the collagen-associated GAGs in rat tendon have been able to shift from CS to DS, which is the predominant PG in the adult tissue (Scott & Orford, 1981). This is probably related to the increase in mechanical strength of the collagen fibrils.

DeKlerk & Human (1985) have studied the GAG content in human fetal and pubertal prostate and have shown that CS is the major GAG of the fetal prostate. It decreases from birth to puberty, while DS is the major GAG at all ages. This may reflect the change in collagen fibrils and their mechanical strength during development. The relative proportion of CSPGs to DSPGs may reflect the degree of tissue saturation or differentiation.

Interstitial PGs

T3 PGs have a wide distribution in the stroma including the interstitial spaces, the reticular layer below the BM, around the bundles of collagen fibrils, and on the surface of fibroblasts. Their distributions

are similar to those reported for prostate gland (Chan & Wong, 1989*a*). Their heavy CB-staining, at high concentrations of $MgCl_2$ (0.3 M) and positive RR-staining at low pH (2.5), show that they are rich in sulphated groups.

It is of interest to note that PGs are more concentrated in stroma with fewer collagen fibrils and lamina propria within the epithelial folds, while they are less numerous in regions where the stroma is more compact and contains large amounts of collagen, especially in regions close to the muscular layer. Similar observations have been noted at the light microscopic level for acidic GAGs by alcian blue at low pH (Chan & Wong, 1991). This would support the view that PGs are important in growth and morphogenesis, and that epithelial cells in alveoli with numerous epithelial folds are more active than cells in alveoli with a distended lumen.

The fine filamentous structure revealed by specimens treated with RR is interesting. Although the nature of these interconnecting filamentous networks is not known, they show the closely linked nature of the stromal PGs among themselves and also with other stromal components such as collagen, basal lamina and stromal cells. These linking filaments may represent hyaluronic acid, and the stromal PGs may form large aggregates or networks by binding to the hyaluronic acid through the link proteins, similar to the well-known structure of the cartilaginous CSPG (Hascall & Hascall, 1981). Corresponding structures have been described in guinea pig prostate (Chan & Wong, 1989*a*), embryonic cornea (Hay, 1978) and aorta (Hascall & Hascall, 1981). The filaments in the cornea are hyaluronidase-sensitive.

CB-positive T3 filaments were removed after treatments with Ch-ABC, Ch-AC, Ch-ABC plus S-hyaluronidase, pronase and 2.0 M $MgCl_2$. They were resistant to nitrous acid, heparitinase, heparinase, neuraminidase and S-hyaluronidase. These suggest that the T3 filaments represent PGs rich in CS.

CSPGs are found in many tissues such as cartilage, bone, intervertebral disc, corneal stroma, ovarian follicular fluid, aorta, skin and some embryonic basement membranes (Heinegard & Paulsson, 1984; Poole, 1986). The functions of the CSPGs in the interstitial spaces are still not fully understood. Due to their relatively large size, they can retain large amounts of water and occupy large hydrodynamic volumes during the hydrated state similar to the cartilaginous CSPG (Hascall & Hascall, 1981). Their irregular occurrence, especially prominent in stroma which is loose in structure and with few collagen fibrils, suggests that they may be involved in some local processes such as growth and regeneration. An increase in HA or CS/DS has been observed in regenerating tendon, liver, ear cartilage and during wound healing (Reid & Flint, 1974; Shetler et al. 1978; Alexander & Donoff, 1980; Edward et al. 1980; Hasty et al. 1981). Hassell et al. (1983) have described a large CS/DSPG component in regenerating cornea. However, this PG disappears when the transparency of the cornea is restored.

The content of CS has been shown to be high in fetal and neoplastic tissues (Sampaio et al. 1977; Chiarugi et al. 1978). An increased amount of CS has been noted in carcinoma of the breast, lung, liver, colon and prostate (Takeuchi et al. 1976; Horai et al. 1981; Kojima et al. 1975, 1982; Iozzo et al. 1982; Iozzo & Wright, 1982; Isemura et al. 1982; DeKlerk & Human, 1984). CS has also been shown to stimulate the growth of mammary carcinoma cells in vitro and Ehrlich ascites tumour cells in vivo, whereas enzymes that degrade CS can retard the growth of the tumour cells (Ozzello et al. 1960; Takeuchi, 1965, 1972). Inhibition of CSPG synthesis by β-D-xyloside has been shown to inhibit branching morphogenesis in mouse fetal salivary glands and kidney tubules (Thompson & Spooner, 1982; Platt et al. 1987). Similar treatment also prohibits the response of the prostate gland and seminal vesicle of the castrated guinea pig to androgen stimulation (Chan & Wong, unpublished observations). Thus stromal PGs may be important in local processes such as cell growth and regeneration.

The fact that T3 PGs were found at the surface of fibroblasts may indicate that they have a very intimate relationship with these cells. Fibroblasts have been suggested as being responsible for PG production (Gallagher et al. 1983), and cell–matrix interaction may be important for cell movement.

Relatively more CB–T3 filaments were noted on the surface of fibroblasts which were in contact with the BM. In vitro studies have indicated that the adhesion sites of newly attached fibroblasts are enriched in both fibronectin and cell-surface PGs (Rollins & Culp, 1979*a*, *b*; Laterra et al. 1980; Woods et al. 1984). It has been shown that fibronectin can interact with the CSPGs at the cell surface of culture fibroblasts (Perkins et al. 1979; Alitalo et al. 1980), and such a binding can enhance the binding of fibronectin to collagen (Dehm & Kefalides, 1978; Crouch et al. 1980; Tryggvason et al. 1980). However, it is not clear at present whether these PGs on the surface of fibroblasts represent newly synthesised PGs, or PGs that are interacting with the cell membrane of the fibroblasts. CSPGs found around collagen fibrils are

thought to have a protective function on collagen fibrils against the action of collagenases and proteases by forming a negatively charged shield (Linares & Laros, 1978). The function of CSPGs in the reticular layer beneath the lamina densa is at present unknown. However, their occurrence was irregular. Like the PGs in the interstitial spaces, they were more abundant in areas where the stroma had a loose network of collagen, whereas in areas where collagen fibrils were densely packed, PGs were sparsely distributed. They may also provide additional support to the basal lamina. It is also possible that those regions of the reticular layer with abundant CSPGs represent less mature regions, or regions with active epithelial cell proliferation. The relationship of CSPG with cell behaviour requires further study.

ACKNOWLEDGEMENTS

We would like to thank Mrs Helen Li-Chan for technical assistance, Mr Johnny Leung for photography and Ms Sandy Fung for secretarial help. This work was supported by HKU CRCG no. 335-031-0015.

REFERENCES

ALEXANDER SA, DONOFF RB (1980) The glycosaminoglycans of open wounds. *Journal of Surgical Research* **29** 422–429.

ALITALO K, VAHERI A, KRIEG T, TIMPE R (1980) Biosynthesis of two subunits of type IV procollagen and of other basement membrane proteins by a human tumor cell line. *European Journal of Biochemistry* **109**, 247–255.

AUSPRUNK DH (1986) Distribution of hyaluronic acid and sulfated glycosaminoglycans during blood vessel development in the chick chorio-allantoic membrane. *American Journal of Anatomy* **177**, 313–331.

BERNFIELD M (1984) Matrix regulation of cell proliferation: implications for growth of the embryo. *Seminars in Perinatology* **8**, 117–122.

BERNFIELD M, BANERJEE SD, KODA JE, RAPRAEGER AC (1984) Remodeling of the basement membrane as a mechanism of morphogenetic tissue interaction. In *The Role of Extracellular Matrix in Development* (ed. R. L. Trelstad), pp. 545–572. New York: Alan R. Liss.

BISSELL MJ, HALL G, PARRY G (1982) How does the extracellular matrix direct gene expression? *Journal of Theoretical Biology* **99**, 31–68.

CHAN L, WONG YC (1989*a*) Ultrastructural localization of proteoglycans by cationic dyes in the epithelial–stromal interface of the guinea pig lateral prostate. *Prostate* **14**, 147–162.

CHAN L, WONG YC (1989*b*) Cytochemical characterization of cuprolinic blue-stained proteoglycans in the epithelial–stromal interface of the guinea pig lateral prostate. *Prostate* **14**, 133–145.

CHAN L, WONG YC (1991) Complex carbohydrate histochemistry of the lateral prostate and seminal vesicle of the guinea pig. *Acta Anatomica*, in press.

CHIARUGI VP, VANNUCCHI S, CELLA C, FIBBI G, DEL ROSSO M, CAPPELLETTI R (1977) Intercellular glycosaminoglycans in normal and neoplastic tissues. *Cancer Research* **38**, 4717–4721.

CIFONELLI JA (1968) Reaction of heparan sulfate with nitrous acid. *Carbohydrate Research* **8**, 233–242.

CLOWES AW, CLOWES MM, GOWN AM, WIGHT TN (1984) Localization of proteoheparan sulfate in rat aorta. *Histochemistry* **80**, 379–384.

COHN RH, BANERJEE SD, BERNFIELD MR (1977) Basal lamina of embryonic salivary gland. Nature of glycosaminoglycan and organization of extracellular materials. *Journal of Cell Biology* **73**, 464–478.

CROUCH Z, SAGE H, BORNSTEIN P (1980) Structural basis for apparent heterogeneity of collagens in human basement membranes: type IV procollagen contains two distinct chains. *Proceedings of National Academy of Sciences, USA* **77**, 745–749.

DANIELSON CC (1982) Mechanical properties of reconstituted collagen fibrils. Influence of a glycosaminoglycan, dermatan sulfate. *Connective Tissue Research* **9**, 219–225.

DEHM P, KEFALIDES NA (1978) The collagenous component of lens basement membrane. The isolation and characterization of chain size collagenous peptide and its relationship to newly synthesized lens components. *Journal of Biological Chemistry* **253**, 6680–6686.

DEKLERK DP (1983) The glycosaminoglycans of normal and hyperplastic prostate. *Prostate* **4**, 73–81.

DEKLERK DP, LEE DV, HUMAN HJ (1984) Glycosaminoglycans of human prostatic cancer. *Journal of Urology* **131**, 1008–1012.

DEKLERK DP, HUMAN HJ (1985) Fluctuations in prostatic glycosaminoglycans during fetal and pubertal growth. *Prostate* **6**, 169–175.

DZIADEK M, FUJIWARA S, PAULSSON M, TIMPL R (1985). Immunological characterization of basement membrane types of heparan sulfate proteoglycan. *EMBO Journal* **4**, 905–912.

EDWARD M, LONG WF, WATSON HHK, WILLIAMSON FB (1980) Sulphated glycosaminoglycans in regenerating rat liver. *Biochemical Journal* **188**, 769–773.

FENGER M, WEWER U, ALBRECHTSEN R (1984) Basement membrane heparan sulfate proteoglycan from the L2 rat yolk sac carcinoma. *FEBS Letters* **173**, 75–79.

FLINT MH, CRAIG AS, REILLY HC, GILLARD GC, PARRY DAD (1984) Collagen fibril diameters and glycosaminoglycan content of skin-indices of tissue maturity and function. *Connective Tissue Research* **13**, 69–81.

FRANSSON LA, HAVSMARK B (1970) Structure of dermatan sulfate. VII. The copolymeric structure of dermatan sulfate from horse aorta. *Journal of Biological Chemistry* **245**, 4770–4783.

FUJIWARA S, WIEDEMANN H, TIMPL R, LUSTIG A, ENGEL J (1984) Structure and interactions of heparan sulfate proteoglycan from a mouse tumor basement membrane. *European Journal of Biochemistry* **143**, 145–157.

GALLAGHER JT, GASIUNAS N, SCHOR SL (1983) Specific association of iduronic acid-rich dermatan sulphate with the extracellular matrix of human skin fibroblasts cultured on collagen gels. *Biochemical Journal* **215**, 107–116.

GORDON JR, BERNFIELD MR (1980) The basal lamina of the postnatal mammary epithelium contains glycosaminoglycans in a precise ultrastructural organization. *Developmental Biology* **74**, 118–135.

HASCALL GK (1980) Cartilage proteoglycans: comparison of sectioned and spread whole molecules. *Journal of Ultrastructural Research* **70**, 369–375.

HASCALL VC, HASCALL GK (1981). Proteoglycans. In *Cell Biology of Extracellular Matrix* (ed. E. D. Hay), pp. 39–63. New York: Plenum Press.

HASSELL JR, ROKEY PG, BARRACH HJ, WILCZEK J, RENNARD SI, MARTIN GR (1980) Isolation of a heparan sulfate-containing proteoglycan from basement membrane. *Proceedings of the National Academy of Sciences, USA* **77**, 4494–4498.

HASSELL JR, CINTRON C, KUBLIN C, NEWSOME DA (1983) Proteoglycan changes during restoration of transparency in corneal scars. *Archives of Biochemistry and Biophysics* **22**, 362–369.

HASSELL JR, LEYSHON WC, LEDBETTER SR, TYREE B, SUZUKI S

(1985) Isolation of two forms of basement membrane proteoglycans. *Journal of Biological Chemistry* **260**, 8098–8105.

HASTY KA, SMITH GN, KANG AH (1981) Studies on glycosaminoglycans of regenerating rabbit ear cartilage. *Developmental Biology* **86**, 198–205.

HAY ED (1978) Fine structure of embryonic matrices and their relation to the cell surface in ruthenium red-fixed tissues. *Growth* **42**, 399–423.

HAY ED (1984) Cell–matrix interaction in the embryo: cell shape, cell surface, cell skeleton, and their role in differentiation. In *The Role of Extracellular Matrix in Development* (ed. R. L. Trelstad), pp. 1–31. New York: Alan R. Liss.

HAYASHI K, HAYASHI M, JALKANEN M, FIRESTONE J, TRELSTAD RL, BERNFIELD MR (1987). Immunocytochemistry of cell surface heparan sulfate proteoglycan in mouse tissues: a light and electron microscopic study. *Journal of Histochemistry and Cytochemistry* **35**, 1079–1088.

HEDMAN K, CHRISTNER J, JULKUNEN L, VAHERI A (1983) Chondroitin sulfate at the plasma membranes of cultured fibroblasts. *Journal of Cell Biology* **97**, 1288–1293.

HEINEGARD D, PAULSSON M (1984) Structure and metabolism of proteoglycans. In *Extracellular Matrix Biochemistry* (ed. K. A. Piez & A. H. Reddi), pp. 277–328. New York: Elsevier.

HOOK M, KJELLER L, JOHANSSON S, ROBINSON J (1984) Cell-surface glycosaminoglycans. *Annual Review of Biochemistry* **53**, 847–869.

HORAI T, NAKAMURA N, TATEISHI R, HATTORIS N (1981) Glycosaminoglycans in human lung. *Cancer* **48**, 2016–2021.

INGBER DE, MADRI JE, FOLKMAN JE (1987) Endothelial growth factors and extracellular matrix regulate DNA synthesis through modulation of cell and nuclear expansion. *In Vitro* **23**, 387–394.

INOUE S, LEBLOND CP (1988) Three-dimensional network of cords: the main component of basement membranes. *American Journal of Anatomy* **181**, 341–358.

IOZZO RV (1985) Proteoglycans: structure, function and role in neoplasia. *Laboratory Investigation* **53**, 373–396.

IOZZO RV, BOLENDER PP, WIGHT TN (1982) Proteoglycan changes in the intercellular matrix of human colon carcinoma: an integrated biochemical and stereologic analysis. *Laboratory Investigation* **47**, 124–138.

IOZZO RV, WIGHT TN (1982) Isolation and characterization of proteoglycans synthesized by human colon and colon carcinoma. *Journal of Biological Chemistry* **257**, 11135–11144.

IOZZO RV, CLARK CC (1986) Biosynthesis of proteoglycans by rat embryo parietal yolk sacs of organ culture. *Journal of Biological Chemistry* **261**, 6658–6669.

IOZZO JT, CLARK CC (1987) Chondroitin sulfate proteoglycan is a constituent of the basement membrane in the rat embryo parietal yolk sac. *Histochemistry* **88**, 23–29.

ISEMURA M, MUNAKATA H, OTATANI N, GOTO K, YOSIZAWA Z (1982) Glycosaminoglycans of rat colorectal adenocarcinoma. *Gann* **73**, 721–727.

KANWAR YS (1984) Biophysiology of glomerular filtration and proteinuria. *Laboratory Investigation* **51**, 7–21.

KANWAR YS, FARQUHAR MG (1979*a*) Anionic sites in the glomerular basement membrane. In vivo and in vitro localization to the laminae rarae by cationic probes. *Journal of Cell Biology* **81**, 137–153.

KANWAR YS, FARQUHAR MG (1979*b*) Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membrane. *Proceedings of the National Academy of Sciences, USA* **76**, 4493–4497.

KANWAR YS, FARQUHAR MG (1979*c*) Presence of heparan sulfate in the glomerular basement membrane. *Proceedings of the National Academy of Sciences, USA* **76**, 1303–1307.

KANWAR YS, LINKER A, FARQUHAR MG (1980) Increased permeability of the glomerular basement membrane to ferritin after removal of heparan sulfate by enzyme digestion. *Journal of Cell Biology* **86**, 688–693.

KANWAR YS, HASCALL VC, FARQUHAR MG (1981) Partial characterization of newly synthesized proteoglycans isolated from the glomerular basement membrane. *Journal of Cell Biology* **90**, 527–532.

KATO M, KOIKE Y, ITO Y, SUZUKI S, KIMATA K (1987) Multiple forms of heparan sulfate proteoglycans in the Engelbreth–Holm–Swarm tumor; the occurrence of high density forms bearing both heparan sulfate and chondroitin sulfate side chains. *Journal of Biological Chemistry* **262**, 7180–7188.

KJELLEN L, PETERSON I, HOOK M (1980) Cell-surface heparan sulfate: an intercalcated membrane proteoglycan. *Proceedings of the National Academy of Sciences, USA* **78**: 5371–5375.

KJELLEN L, OLDBERG A, HOOK M (1981) Cell surface heparan sulfate: mechanisms of proteoglycan–cell association. *Journal of Biological Chemistry* **255**, 10407–10413.

KOFOED JA, HOUSSAY AB, TOCCI AA, CLURBELO HM, GAMPER CH (1971) Effects of testosterone on glycosaminoglycans in the prostate, seminal vesicles and salivary glands of the rat. *Journal of Endocrinology* **51**, 465–471.

KOJIMA J, NAKAMURA N, KANATANI M, OHMORI K (1975) The glycosaminoglycans in human hepatic cancer. *Cancer Research* **35**, 542–547.

KOJIMA J, NAKAMURA N, KANATANI M, AKIYAMA M (1982) Glycosaminoglycans in 3′-methyl-4-dimethyl aminoazo benzene-induced rat hepatic cancer. *Cancer Research* **42**, 2857–2860.

LATERRA J, ANSBACHER R, CULP LA (1980) Glycosaminoglycans that bind cold-insoluble globulin in cell-substratum adhesion sites of murine fibroblasts. *Proceedings of the National Academy of Sciences, USA* **77**, 6662–6666.

LAURIE GW, LEBLOND CP, INOUE S, MARTIN GR, CHUNG A (1984) Fine structure of the glomerular basement membrane and immunolocalization of five basement membrane components to the lamina densa (basal lamina) and its extension in both glomeruli and tubules of the rat kidney. *American Journal of Anatomy* **169**, 463–481.

LAURIE GW (1985) Lack of heparan sulfate proteoglycan in a discontinuous and irregular placental basement membrane. *Developmental Biology* **108**, 299–309.

LERNER L, TORCHIA DA (1986) A multinuclear study of the interactions of cations with proteoglycans, heparin, and ficoll. *Journal of Biological Chemistry* **261**, 12706–12714.

LI ML, AGGELER J, FARSON DA, HATIER C, HASSELL J, BISSELL MJ (1987) Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proceedings of the National Academy of Sciences, USA* **84** 136–140.

LINARES HA, LAROS DL (1978) Proteoglycans and collagen in hypertrophic scar formation. *Plastic Reconstruction Surgery* **62**, 589–593.

LINKER A, HOVINGH P (1972) Heparinase and heparitinase from Flavobacteria. In *Methods in Enzymology*, vol. 28 (ed. V. Ginsberg), pp. 902–911. New York: Academic Press.

MALMSTROM A, FRANSSON LA, HOOK M, LINDAHL U (1975) Biosynthesis of dermatan sulfate. I. Formation of the L-iduronic acid residues. *Journal of Biological Chemistry* **250**, 3419.

MARTIN GR, KLEINMAN HK, TERRANOVA VP, LEDBETTER S, HASSELL JR (1984) The regulation of basement membrane formation and cell–matrix interactions by defined supramolecular complexes. *Ciba Foundation Symposium* **108**, 197–212.

MARTIN GR, TIMPL R (1987) Laminin and other basement membrane components. *Annual Review of Cell Biology* **3**, 57–85.

MARTINEZ-HERNANDEZ A, AMENTA PS (1983) The basement membrane in pathology. *Laboratory Investigation* **48**, 656–677.

MCCARTHY KJ, ACCAVITTI MA, COUCHMAN JR (1989) Immunological characterization of a basement membrane-specific chondroitin sulfate proteoglycan. *Journal of Cell Biology* **109**, 3187–3198.

NORDLING B, GLIMELIUS B, WASTESON A (1981) Heparan sulfate proteoglycan of cultured cells: demonstration of a lipid- and a

matrix-associated form. *Biochemical and Biophysical Research Communication* **103**, 1265–1272.

OBRINK B (1973) A study of the interactions between monomeric tropocollagen and glycosaminoglycans. *European Journal of Biochemistry* **33**, 387–400.

OBRINK B (1981) The influence of glycosaminoglycans on the formation of fibres from monomeric tropocollagen in vitro. *European Journal of Biochemistry* **34**, 129–137.

OBRINK B, PERTOFT H, IVERIUS PH, LAURENT TC (1975) The effect of calcium on the macromolecular properties of heparan sulfate. *Connective Tissue Research* **3**, 187–193.

OLDBERG A, KJELLEN L, HOOK M (1979) Cell-surface heparan sulfate. Isolation and characterization of a proteoglycan from rat liver membranes. *Journal of Biological Chemistry* **254**, 8505–8510.

OOHIRA A, WIGHT TN, MCPHERSON J, BORNSTEIN P (1982) Biochemical and ultrastructural studies of proteoheparan sulfates synthesized by PYS-2, a basement membrane-producing cell line. *Journal of Cell Biology* **92**, 357–367.

OZZELLO L, LASFARGUES EY, MURRAY MR (1960) Growth-promoting activity of acid mucopolysaccharides on a strain of human mammary carcinoma cells. *Cancer Research* **20**, 600–604.

PARMLEY RT, TAKAGI M, DENYS FR (1984) Ultrastructural localization of glycosaminoglycans in human term placenta. *Anatomical Record* **210**, 477–484.

PARTHASARATHY N, SPIRO RG (1981) Characterization of the glycosaminoglycan component of the renal glomerular basement membrane and its relationship to the peptide portion. *Journal of Biological Chemistry* **256**, 507–513.

PAULSSON M, DZIADEK M, SUCHANEK C, HUTTNER WB, TIMPL R (1985) Nature of sulphated macromolecules in mouse Reichert's membrane. Evidence for tyrosine *O*-sulphate in basement-membrane proteins. *Biochemical Journal* **231**, 571–579.

PAULSSON M, FUJIWARA S, DZIADEK M, TIMPL R, PEJLER G, BACKSTROM G, LINDAHL U, ENGEL J (1986) Structure and function of basement membrane proteoglycans. *Ciba Foundation Symposium* **124**, 189–203.

PLATT JL, BROWN DM, GRANLUND K, OEGEMA TR, KLEIN DJ (1987) Proteoglycan metabolism associated with mouse metanephric development: morphological and biochemical effects of β-D-xyloside. *Developmental Biology* **123**, 293–306.

PERKINS ME, JI TH, HYNES RO (1979) Crosslinking of fibronectin to sulfated proteoglycans at the cell surface. *Cell* **16** 941–952.

POOLE AR (1986) Proteoglycans in health and disease: structures and functions. *Biochemical Journal* **236**, 1–14.

RAPRAEGER AC, BERFIELD MR (1982) An integral membrane proteoglycan is capable of binding components of the cytoskeleton and the extracellular matrix. In *Extracellular Matrix* (ed. S. Hawkes & J. L. Wang), pp. 265–268. New York: Academic Press.

RAPRAEGER AC, BERNFIELD MR (1985) Cell surface proteoglycan of mammary epithelial cells. *Journal of Cell Biological Chemistry* **260**, 4103–4109.

RAPRAEGER AC, JALKANEN M, ENDO E, KODA J, BERNFIELD MR (1985) The cell surface proteoglycan from mouse mammary epithelial cells bears chondroitin sulfate and heparan sulfate glycosaminoglycans. *Journal of Biological Chemistry* **160**, 11046–11052.

RAPRAEGER A, JALKANEN M, BERNFIELD M (1986) Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells. *Journal of Cell Biology* **103**, 2683–2696.

REDDI AH (1984) Extracellular matrix and development. In *Extracellular Matrix Biochemistry* (ed. K. A. Piez & A. H. Reddi), pp. 375–412.New York: Elsevier.

REID T, FLINT MH (1974) Changes in glycosaminoglycan content on healing rabbit tendon. *Journal of Embryology and Experimental Morphology* **31**, 489–495.

ROBERTS R, GALLAGHER J, SPOONCER E, ALLEN TD, BLOOMFIELD F, DEXTER TM (1988) Heparan sulfate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature* **332**, 376–378.

ROLLINS BJ, CULP LA (1979*a*) Glycosaminoglycans in the substrate adhesion sites of normal and virus transformed murine cells. *Biochemistry* **18**, 141–148.

ROLLINS BJ, CULP LA (1979*b*) Preliminary characterization of the proteoglycans in the substrate adhesion sites of normal and virus-transformed murine cells. *Biochemistry* **18**, 5621–5629.

RUGGERI A, DELLORBO C, QUACCI D (1975) Electron microscopic visualization of proteoglycans with alcian blue. *Histochemical Journal* **7**, 187–197.

RZESZOWSKA G (1966) Histochemical analysis of acid mucopolysaccharides in the epithelium of the seminal vesicles and gonads in the postembryonic period. *Folia Morphologica* **25**, 436–442.

SAMPAIO LO, DIETRICH CP, FILHO OG (1977) Changes in sulfated mucopolysaccharide composition of mammalian tissues during growth and in cancer tissues. *Biochemica et Biophysica Acta* **498**, 123–31.

SAITO H, YAMAGATA T, SUZUKI S (1968) Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. *Journal of Biological Chemistry* **243**, 1536–1542.

SATO CS, GYORKEY F (1972) Glycosaminoglycans of normal human prostate. *Proceedings of the Society of Experimental Biology and Medicine* **140**, 79–83.

SCHOFIELD BH, WILLIAMS BR, DOTY SB (1975) Alcian blue staining of cartilage for electron microscopy. Application of the critical electrolyte concentration principle. *Histochemical Journal* **7**, 139–149.

SCOTT JE (1980) Collagen–proteoglycan interaction. Localization of proteoglycan in tendon by electron microscopy. *Biochemical Journal* **187**, 887–891.

SCOTT JE (1985) Proteoglycan histochemistry: a valuable tool for connective tissue biochemists. *Collagen Related Research* **5**, 541–575.

SCOTT JE (1988) Proteoglycan–fibrillar collagen interactions. *Biochemical Journal* **252**, 313–323.

SCOTT JE, ORFORD CR (1981) Dermatan sulphate rich proteoglycan associates with rat tail-tendon collagen at the d band in the gap region. *Biochemical Journal* **197**, 213–216.

SCOTT JE, ORFORD CR, HUGHES EW (1981) Proteoglycan–collagen arrangement in developing rat tendon. *Biochemical Journal* **195**, 573–581.

SHETLAR MR, DAVITT WF, SHETLAR CL, ROSETT RL, CRASS MF, LAUTSCH EV, KISCHER CW (1978) Glycosaminoglycan changes in healing myocardial infarction. *Proceedings of the Society of Experimental Biology and Medicine* **158**, 210–214.

SIMIONESCU M, SIMIONESCU N, PALADE GE (1984) Partial chemical characterization of the anionic sites in the basal lamina of fenestrated capillaries. *Microvascular Research* **28**, 352–367.

SPOONER BS, PAULSEN A (1986) Basal lamina anionic sites in the embryonic submandibular salivary gland: resolution and distribution using ruthenium red and polyethyleneimine as cationic probes. *European Journal of Cell Biology* **41**, 230–237.

TAKEUCHI J (1965) Growth promoting effect of chondroitin sulfate on solid Ehrlich ascites tumor. *Nature* **207**, 537–538.

TAKEUCHI J (1972) Effect of chondroitinases on the growth of solid Ehrlich ascites tumor. *British Journal of Cancer* **26**, 115–119.

TAKEUCHI J, SOKUE M, SATO E, SHAMOTO M, MUIRA K, NAKAGAKI S (1976) Variation in glycosaminoglycan components of breast tumors. *Cancer Research* **36**, 2133–2139.

THOMAS KA, GIMENEZ-GALLEGO G (1986) Fibroblast growth factors: broad spectrum mitogens with potent angiogenic activity. *Trends in Biochemical Sciences* **11**, 81–84.

THOMPSON HA, SPOONER BS (1982) Inhibition of branching morphogenesis and alteration of glycosaminoglycan biosynthesis in salivary glands treated with β-D-xyloside. *Developmental Biology* **89**, 417–424.

TOOLE BP (1981) Glycosaminoglycans in morphogenesis. In *Cell*

Biology of Extracellular Matrix (ed. E. D. Hay), pp. 259–288. New York: Plenum.

TRELSTAD RL, HAYASHI K, TOOLE BP (1974) Epithelial collagens and glycosaminoglycans in the embryonic cornea. Macromolecular order and morphogenesis in the basement membrane. *Journal of Cell Biology* **62**, 815–830.

TRYGGVASON K, ROKEY PG, MARTIN GR (1980) Biosynthesis of the type IV procollagens. *Biochemistry* **19**, 1284–1289.

VACCARO CA, BRODY JS (1979) Ultrastructural localization and characterization of proteoglycans in the pulmonary alveolus. *American Review of Respiratory Diseases* **120**, 901–910.

VAN KUPPEVELT THMSM, DOMEN JGW, CREMERO FPM, KUYPER CMA (1984*a*) Staining of proteoglycans in mouse lung alveoli. I. Ultrastructural localization of anionic sites. *Histochemical Journal* **16**, 657–669.

VAN KUPPEVELT THMSM, DOMEN JGW, CREMERO FPM, KUYPER CMA (1984*b*) Staining of proteoglycans in mouse lung alveoli. II. Characterization of the cuprolinic blue-positive, anionic sites. *Histochemical Journal* **16**, 671–686.

VAN KUPPEVELT THMSM, CREMERO FPM, DOMEN JGW, VAN BENUNINGEN H, VAN DEN BRULE AJC, KUYPER CMA (1985) Ultrastructural localization and characterization of proteoglycans in human lung alveoli. *European Journal of Cell Biology* **36**, 74–80.

VOGEL KG, PAULSSON M, HEINEGARD D (1984) Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. *Biochemical Journal* **233**, 587–597.

WOODS A, HOOK M, KJELLEN L, SMITH CG, REES DA (1984) Relationship of heparan sulfate proteoglycans to the cytoskeleton and extracellular matrix of cultured fibroblasts. *Journal of Cell Biology* **99**, 1743–1753.

YURCHENCO PD, TSILIBARY EC, CHARONIS AS, FURTHMAYR H (1987) Models for the self assembly of basement membrane. *Journal of Histochemistry and Cytochemistry* **34**, 93–102.